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Osteopontin regulation in cultured rat renal epithelial cells

URIEL M. MALYANKAR, MANUELA ALMEIDA, RICHARD J. JOHNSON, RAIMUND H. PICHLER, and CECILIA M. GIACHELLI

Department of Pathology and Department of Nephrology, University of Washington, Seattle, Washington, USA

Osteopontin regulation in cultured rat renal epithelial cells. Osteopontin is a secreted, arginine-glycine-aspartate (RGD)-containing phosphoprotein that is up-regulated in kidney cortical tubular epithelial cells in many experimental models of tubulointerstitial fibrosis. Its close association with infiltrating macrophage in this disease and its ability to directly stimulate macrophage migration has made it a key target as a molecule likely to be important in mediating renal inflammation. The mechanism responsible for osteopontin up-regulation in kidney disease is unknown, but may involve induction by specific cytokines released by damaged glomeruli or other parts of the kidney, prior to the onset of interstitial disease. We have investigated this hypothesis by testing the effects of angiotensin II, bFGF, TGFβ1, EGF, and IGF, important renal cytokines, on osteopontin regulation in cultured NRK52E cells, a rat renal epithelial cell line. Using Northern blot, Western blot, and ELISA analyses, we find that NRK52E cells constitutively express low levels of osteopontin mRNA and protein. TGFβ1 and EGF are potent inducers of osteopontin mRNA and protein in these cells. mRNA stability and nuclear run on assays suggest that induction of osteopontin expression by TGFβ1 and EGF is via increased transcription of the osteopontin gene. In contrast, IGF-1, angiotensin II, and PDGF BB did not significantly modulate osteopontin expression in NRK52E cells. These studies are consistent with the hypothesis that release of potent cytokines by the injured kidney might be one mechanism whereby elevated levels of osteopontin are synthesized by cortical tubular epithelial cells early in tubulointerstitial disease.

Tubulointerstitial disease accompanies most types of glomerular and extraglomerular renal diseases, and is characterized by infiltration of mononuclear cells, atrophy and dilation of tubules, and increased deposition of collagen in the interstitium [1]. A growing number of studies suggest that leukocyte accumulation in the interstitium plays a major causal role in development of this disease [2–5]. The importance of the tubulointerstitial injury has been underscored by studies that have demonstrated that the severity of the tubulointerstitial disease correlates with both the degree of renal impairment and risk of progression to end-stage kidney failure [6, 7]. Thus, determining the mechanisms that control the development of tubulointerstitial fibrosis is of major importance.

We have recently focused on osteopontin as a potential regulator of tubulointerstitial injury. Osteopontin is an RGD-containing acidic glycoprotein with cell adhesive and chemotactic properties *in vitro* [reviewed in 8]. In the kidney, osteopontin is normally made by the descending thin limb of the loop of Henle and some collecting ducts of the medulla, but is virtually absent from renal cortical or juxtaglomerular tubules [9]. Osteopontin is found in urine where it may act as an inhibitor of calcium oxalate crystal growth [10]. In contrast, osteopontin levels are strikingly elevated in experimental models of renal disease associated with cortical tubulointerstitial fibrosis [9, 11]. Osteopontin has also been shown to be elevated in animal models of tubulointerstitial disease caused by cyclosporine, protein overload, and unilateral ureteral obstruction [12–14]. In all cases, a striking association of osteopontin with sites of macrophage accumulation was noted. *In vivo* and *in vitro*, osteopontin is a chemotactic and adhesive factor for macrophages [15–17], suggesting that osteopontin might play a role in modulating the inflammatory response observed under these conditions.

However, given the multiplicity of its receptors and diverse functions, osteopontin might play multiple roles during renal injury and repair. For example, osteopontin levels were enhanced in tubular epithelial cells following IGF-1 pretreatment in ischemic rat kidneys [18]. Since IGF-1 accelerated the recovery of normal renal function and morphology post-ischemia [19], this suggested that osteopontin might serve to promote tissue regeneration and tissue remodeling after acute ischemic injury of the kidney. In this context, it is interesting that osteopontin levels were decreased in human transplant kidneys that showed rejection compared to nonrejecting kidneys, suggesting that osteopontin levels might be important to the success of kidney engraftment [20]. In support of this concept, osteopontin has been shown to inhibit cytokine-induced iNOS synthesis and nitric oxide production in human tubular epithelial cells suggesting a possible cell protective function [21]. Regardless of its functions, the fact that diverse injuries to the kidney results in osteopontin elevation suggests that: (1) osteopontin might be an important common mediator of renal injury and repair and (2) osteopontin synthesis is tightly regulated in the kidney.

Despite the overwhelming number of studies showing the association of osteopontin with renal disease, we know very little about the regulation of osteopontin in kidney epithelial cells. We have hypothesized that cytokines released by the injured kidney might be responsible for the observed changes in osteopontin expression following injury [22]. The purpose of the present study was to identify regulators of osteopontin expression in renal epithelial cells focusing on cytokines that have been implicated in renal interstitial disease. Therefore, we examined the effect of various cytokines on osteopontin mRNA and protein synthesis in NRK52E cells, a well characterized rat renal epithelial cell line.

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Our studies suggest that TGFβ1 and EGF are potent inducers of osteopontin synthesis in NRK52E cells. These cytokines appear to act predominantly via transcriptional mechanisms to regulate osteopontin synthesis in renal epithelial cells.

Methods

Cells

A normal rat kidney epithelial cell line, NRK52E [23], was obtained from ATCC at passage 15 and cultured in DMEM-high glucose media containing 10% calf serum. These cells have previously been shown to secrete C-type natriuretic peptide [24], contain EGF [23, 25] and TGFβ receptors [26].

Cytokine treatment

Cells were grown to confluence and treated on day 0 with the following cytokines or vehicle alone in serum-free media: Angiotensin II (1 μM; Sigma, St. Louis, MO, USA), bFGF (10 ng/ml; Collaborative Research, Bedford, MA, USA), TGFβ1 (10 ng/ml; a gift from Dr. Purchio, Bristol-Meyers Squibb Pharmaceutical Research Institute, Seattle, WA, USA), PDGF-BB (10 ng/ml; a gift from Dr. Charlie Hart, Zymogenetics, Seattle, WA, USA), EGF (50 ng/ml; R & D Systems, Minneapolis, MN, USA), and IGF-1 (50 ng/ml; R & D Systems).

Northern blot analysis

Total RNA was isolated using the TRIZOL method (Gibco, Grand Island, NY, USA) and electrophoresed in a denaturing 1% agarose gel as previously described [27]. After transfer to Zeta-Probe, the filters were probed with ^{32}P-labeled 2B7, a rat osteopontin cDNA, as previously described [27]. Data were normalized to 28S ribosomal RNA [28]. Quantitation was performed using the phosphorimager facility of the Markey Molecular Medicine Center at the University of Washington.

Western blot and ELISA analyses

Conditioned medias were collected from each treatment group and protein content measured using the microBCA assay. Twenty microliters of each were electropheresed on a 10% SDS-polyacrylamide gel, transferred and blotted for osteopontin using MPIIIB10 [1] (obtained from the Developmental Studies Hybridoma Bank maintained under NICHD contract N01-HD-6-2915) as previously described [29]. A second 20 μg aliquot was analyzed by ELISA using MPIIIB10(1) as the capture antibody and OP199, an anti-rat osteopontin polyclonal antibody [30], as the detection antibody. Wells of a Nunc maxisorp plate were coated with 1 μg MPIIIB10(1) in 50 μl PBS for two hours at room temperature. Wells were washed with PBS and then incubated with blocking solution (2% BSA/1% normal rabbit serum in PBS). After washing, conditioned media containing 20 μg of total protein, or known concentration of purified rat osteopontin, were added to the wells and incubated for two hours at room temperature, or overnight at 4°C. After washing with PBS, 250 ng OP199 was added in blocking solution and incubated at room temperature for one hour. After washing with PBS, 50 μl of ABC solution (Vectastain Elite ABC kit; Vector, Burlingame, CA, USA) was added and incubated for 45 minutes at room temperature. After washing with PBS, color was developed using the chromogenic substrate o-phenylenediamine. Reactions were stopped with 100 μl of 4.5 M sulfuric acid. Absorbance was measured at 490 nm, and concentrations of osteopontin determined by comparison to a standard curve generated using purified rat osteopontin [30] on the same plate.

Actinomycin D treatment

Cells were treated with EGF and TGFβ1 as described above for 72 hours. Actinomycin D (12.5 μg/ml) was added to the medium and total RNA was isolated at different time points following treatment. Northern blot analysis, normalization, and quantitation were carried out as described above.

Nuclear run-on assays

Nuclear run-on assays were performed using a modification of previously described protocols [31, 32]. Cells were treated with EGF and TGFβ1 as described above and nuclei were isolated as described previously [32]. The run-on reaction was carried out as described previously except that the α(^{32}P)-UTP labeled RNA was purified using the Trizol method. Labeled RNA was hybridized to 5 μg of linearized plasmid DNA that had been previously bound to Zetaprobe membrane (Bio-Rad, Hercules, CA, USA). Plasmid DNAs used were 2B7 (osteopontin), pBA-1 (gamma actin cDNA which cross reacts with alpha, beta and gamma actin [33]), and control plasmid containing no insert, pUC7 (NEB, Beverly, MA, USA). Equal amounts of labeled RNAs (5×10^6 cpm) were added to the membranes. Hybridization was carried out at 65°C for 36 hours in hybridization buffer (0.25 M Na_2HPO_4, pH 7.2 and 7% SDS). The membranes were washed at 65°C under conditions of high stringency (20 mM Na_2HPO_4, pH 7.2 and 5% SDS twice for 20 min each; 20 mM Na_2HPO_4, pH 7.2 and 1% SDS twice for 20 min each) and quantitated using a phosphorimager.

Results

Osteopontin mRNA levels

In order to examine regulators of osteopontin expression in rat kidney epithelial cells, NRK52E cells were treated with a variety of renal cytokines that have been implicated in renal disease. As shown in Figure 1, NRK52E cells constitutively express low levels of osteopontin mRNA. Treatment of cells with TGFβ1 and EGF increased osteopontin mRNA levels within a 24 hour period. Levels of mRNA increased at 48 hours and were highest at 72 hours following treatment. TGFβ1 was the strongest inducer, showing an ~8.5-fold induction of osteopontin mRNA at 72 hours followed by EGF that showed an ~7.0-fold induction at 72 hours. In contrast, IGF-1, bFGF, PDGF BB, and angiotensin II did not appreciably alter osteopontin expression levels in NRK52E cells, even though optimal receptor binding concentrations of cytokines were used. Similar results were obtained in a second independent experiment (not shown).

Osteopontin protein levels

In order to determine whether increased steady state levels of osteopontin mRNA translated into increased osteopontin synthesized and secreted by NRK52E following cytokine treatment, we performed Western blot analysis of conditioned media samples. As shown in Figure 2A, very little osteopontin protein was normally secreted by NRK52E cells, although small amounts could be seen on prolonged exposure of the blot (not shown). This was consistent with the low mRNA levels observed by Northern blot analysis (Fig. 1). Addition of either EGF or TGFβ1 elevated

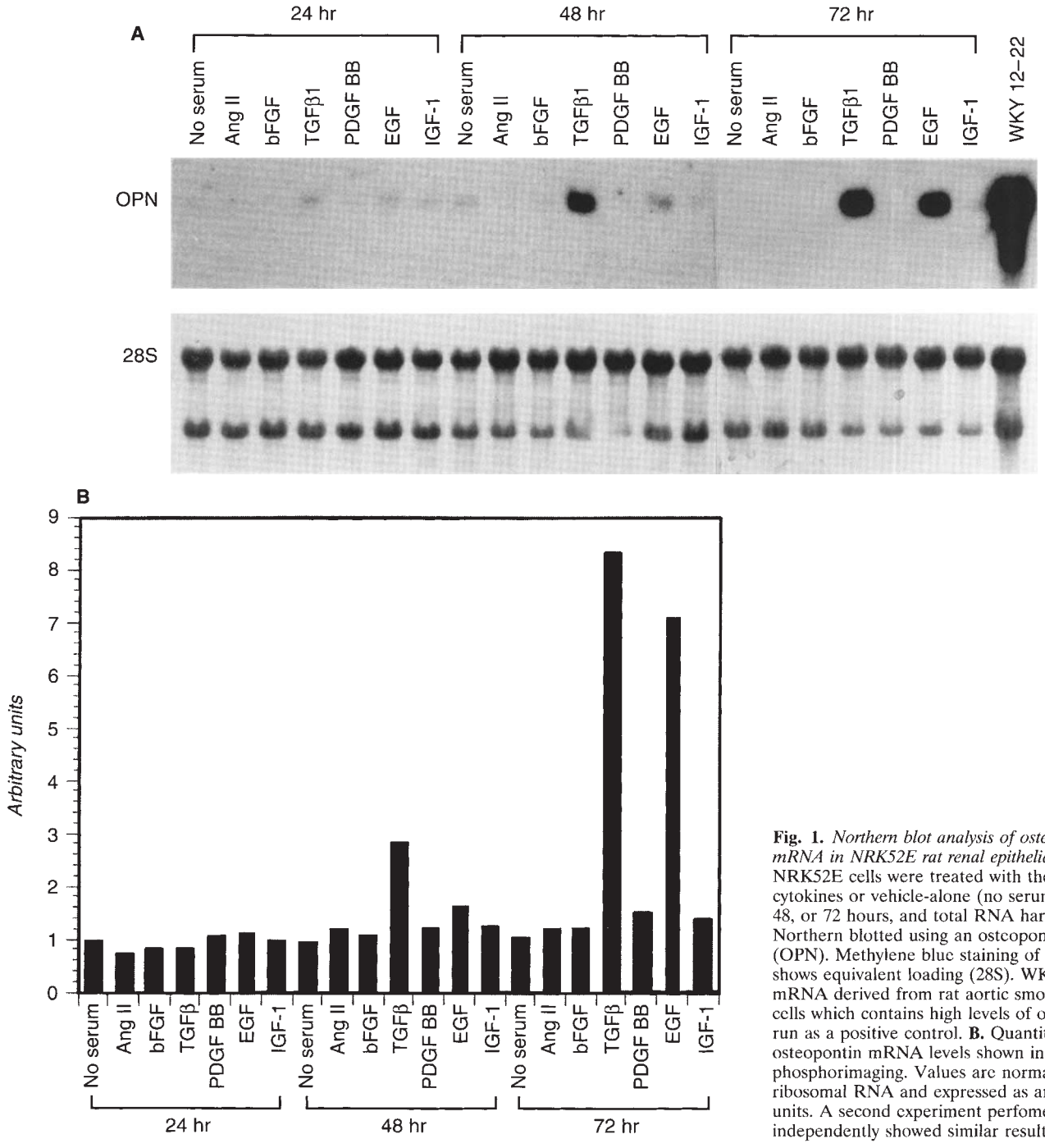

Fig. 1. *Northern blot analysis of osteopontin mRNA in NRK52E rat renal epithelial cells.* **A.** NRK52E cells were treated with the indicated cytokines or vehicle-alone (no serum) for 24, 48, or 72 hours, and total RNA harvested and Northern blotted using an osteopontin cDNA (OPN). Methylene blue staining of the filter shows equivalent loading (28S). WKY12-22, mRNA derived from rat aortic smooth muscle cells which contains high levels of osteopontin run as a positive control. **B.** Quantitation of osteopontin mRNA levels shown in (A) by phosphorimaging. Values are normalized to 28S ribosomal RNA and expressed as arbitrary units. A second experiment perfomed independently showed similar results.

osteopontin protein level in the media of NRK52E cells with highest levels seen at two and three days after treatment. The predominant form of osteopontin observed under these conditions had an apparent molecular weight of 66 kDa, consistent with the size previously reported in rat kidney and smooth muscle cells [9, 29]. However, in the TGFβ1 treated cells, an additional immunoreactive band was observed at 68 kDa. We suspect that this band may represent a differentially phosphorylated, glycosylated, or spliced isoform of osteopontin. No increase in osteopontin protein was seen in cells treated with IGF-1, PDGF BB, angiotensin II or bFGF (data not shown). Equivalent loading was demonstrated by Coomassie staining of a duplicate gel (Fig. 2B).

To obtain more precise quantitation of the levels of OPN secreted by these cells under various conditions, we developed a sandwich ELISA for rat osteopontin. Two different antibodies were tested for their ability to act as capture or detection antibodies for this assay: OP199 and MPIIIB10(1). As shown in Figure 3A, the sandwich ELISA was most quantitative if MPIIIB10(1) was used as the capture antibody (coated on the wells) and OP199 used as the detection antibody. Using these

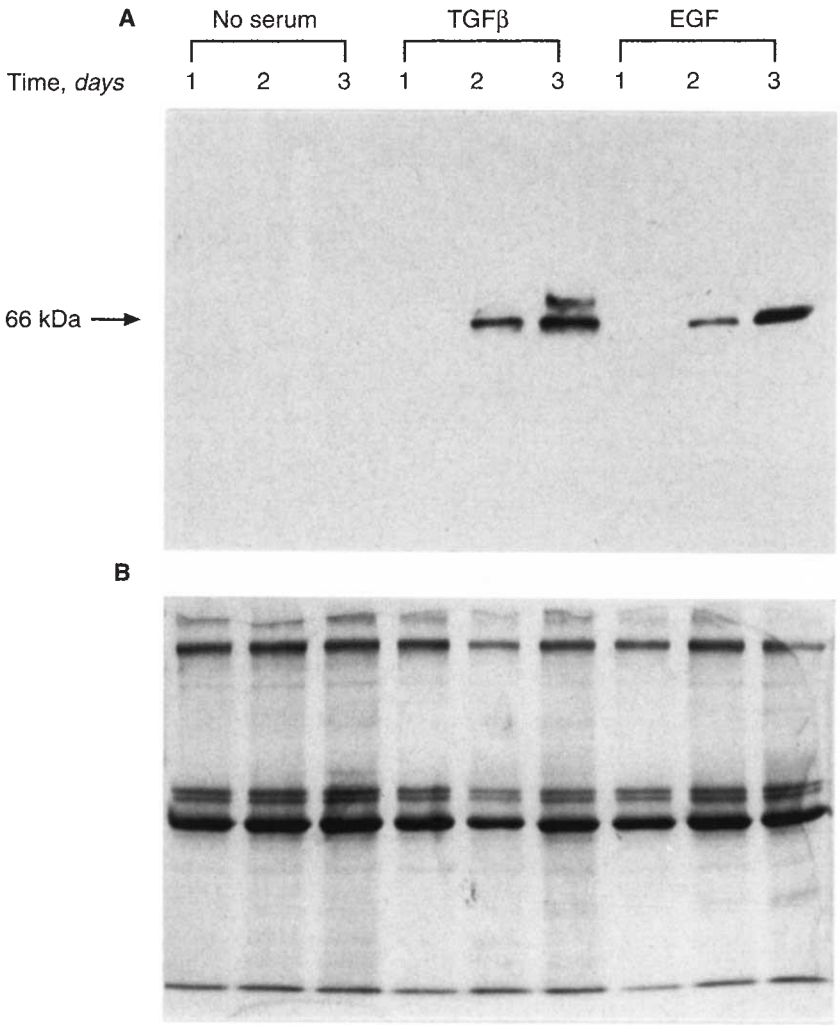

Fig. 2. *Western blot of osteopontin protein secreted by NRK52E rat renal epithelial cells.* NRK52E cells were treated with TGFβ1, EGF or no serum, for 1, 2, or 3 days and conditioned media were collected, electrophoresed, and either Western blotted using anti-rat osteopontin antibody, MPIIIB10(1) (**A**) or stained with Coomassie Blue (**B**). A second experiment performed independently showed similar results (not shown).

conditions, a sensitive ELISA with a linear range spanning 1 to 20 ng/well was developed (Fig. 3B).

To confirm the utility of this assay, we performed sandwich ELISA analysis on the samples examined by Western blot analysis in Figure 2A. As shown in Figure 4, we found that NRK52E cells secrete very little osteopontin protein (< 0.1 μg/ml) normally, whereas treatment with TGFβ1 increased levels to 1.4 μg/ml at 48 hours and 2 μg/ml at 72 hours. Conditioned media from EGF treated cells contained 0.45 μg/ml after 48 hours and 0.9 μg/ml after 72 hours of treatment. These data are qualitatively in good agreement with the relative amounts of osteopontin observed by Western blot analysis of the same samples (Fig. 2).

Osteopontin mRNA stability

To explore the mechanism of regulation of the osteopontin gene by cytokines in kidney epithelial cells, we performed mRNA stability and transcription analyses using the NRK52E cells. To do this we chose to focus on EGF and TGFβ1 treatments, since these produced the greatest increases in osteopontin expression in this cell type.

The increased steady-state levels of osteopontin mRNA induced by TGFβ1 and EGF in NRK52E cells might be due to stabilization of the osteopontin mRNA by TGFβ1, increased transcription of the osteopontin gene, or both. To examine the half-life of osteopontin mRNA, we first treated cells with TGFβ1 or EGF for 72 hours to induce maximum osteopontin mRNA levels, and then treated the cells with actinomycin D to block further mRNA synthesis. We then measured osteopontin mRNA decay with time by Northern blot analysis. As shown in Figure 5 osteopontin mRNA is quite stable in cells treated with EGF as well as the control cells, the half life of the osteopontin mRNA in both cases being > 12 hours. On the other hand, TGFβ1 induced a dramatic de-stabilization of the osteopontin mRNA. The half life of osteopontin mRNA in the TGFβ1 and Actinomycin D treated cells was ~one hour. These data suggest that changes in osteopontin mRNA stability do not account for the increases in steady-state mRNA levels seen after treating these cells with EGF and TGFβ1. On the contrary, because of the enhanced turnover of osteopontin mRNA in the TGFβ1 treated cells, very high or sustained transcription of the osteopontin gene appears to be required for the induction of osteopontin mRNA under these conditions.

Osteopontin gene transcription

Since a change in mRNA stability did not appear to account for the changes in steady state levels of osteopontin mRNA following

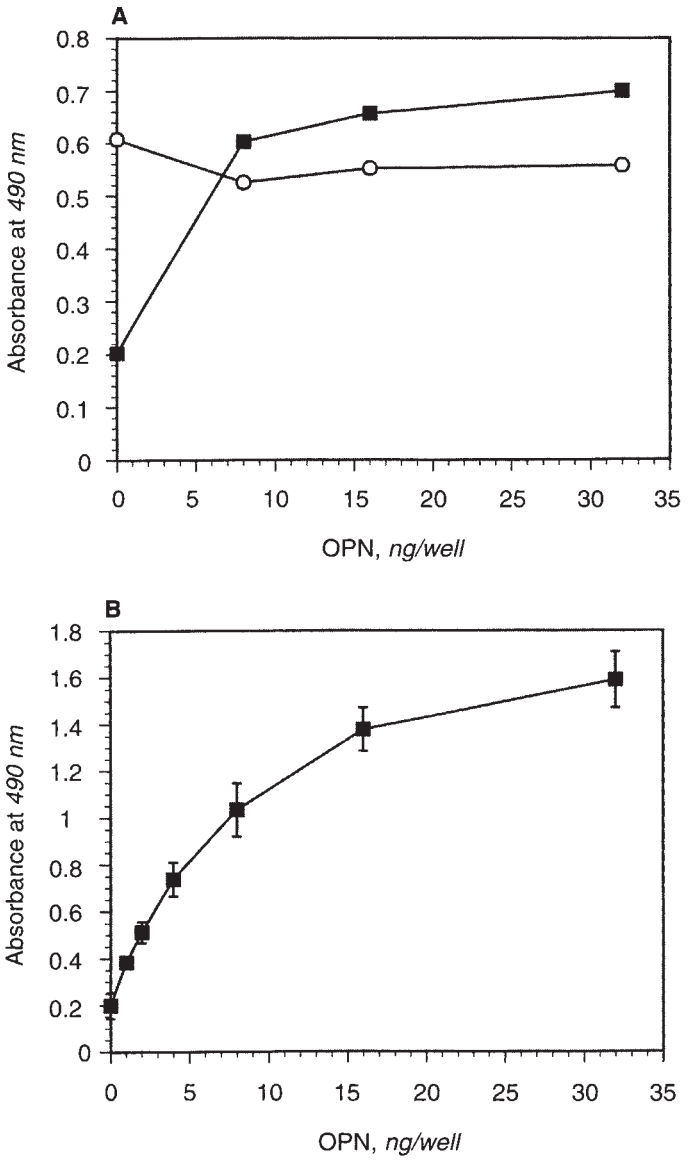

Fig. 3. *ELISA development for rat osteopontin quantitation.* **A.** Microtiter wells were coated with either MPIIIB10(1) or OP199, and ELISA analysis performed using OP199 or MPIIIB10(1) as the detection antibody. Symbols are: (■) MPIIIB10(1) as capture antibody with OP199 as detection antibody; (○) OP199 as capture antibody with MPIIIB10(1) as detection antibody. Points represent average of duplicates. **B.** ELISA standard curves using MPIIIB10(1) as capture antibody and OP199 as detection antibody. Purified rat smooth muscle derived osteopontin was used as the standard antigen. Each point represents the average of triplicates ± SD.

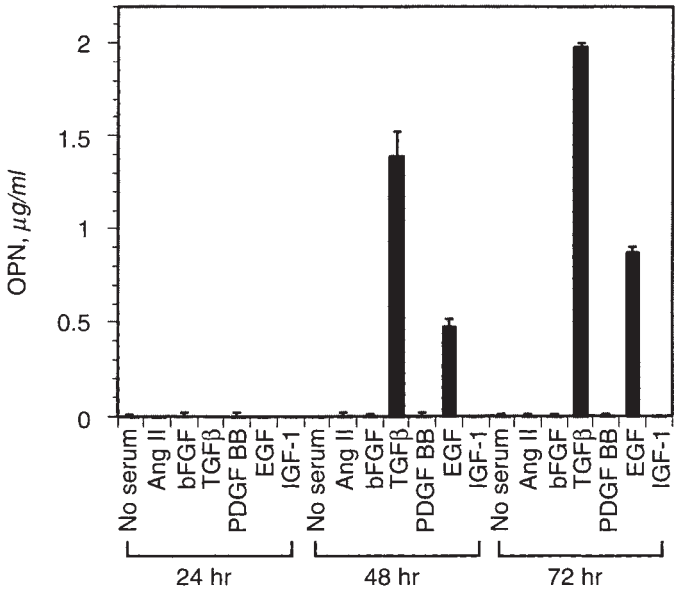

Fig. 4. *Quantitation of osteopontin protein secreted by NRK52E cells.* ELISA analysis was performed on NRK52E cell conditioned media 24, 48, and 72 hours following treatment with the indicated cytokines or vehicle alone (no serum). Osteopontin levels are expressed as μg/ml conditioned media. Each point represents the average of triplicates ± SD.

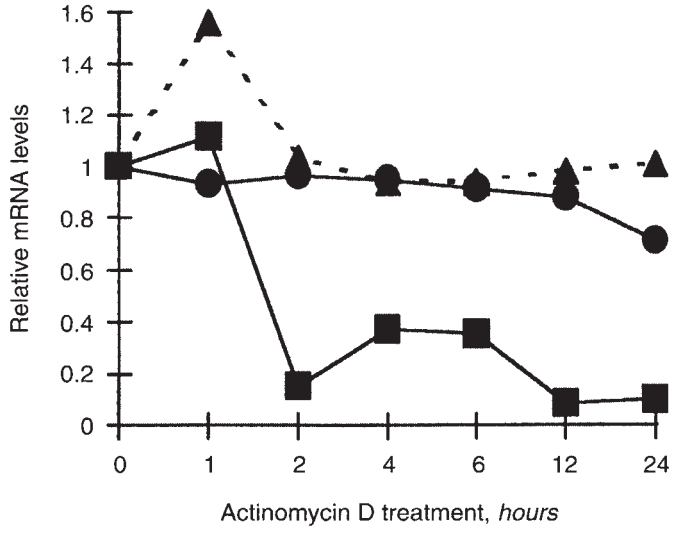

Fig. 5. *Effect of TGFβ1 and EGF on osteopontin mRNA stability.* NRK52E cells were treated with the cytokines or control (no cytokines) for 72 hours and then treated with actinomycin D (12.5 μg/ml) for different time points. Total RNA was harvested and used for Northern blot analysis. RNA levels were normalized using 28S RNA and arbitrary values were plotted relative to the zero time point (relative mRNA levels). Symbols are: (▲) control; (●) EGF; (■) TGFB.

cytokine treatment, we performed nuclear run on assays to measure transcription rate of the osteopontin gene. As shown in Figure 6, transcription of the osteopontin gene was increased by both TGFβ1 and EGF in NRK52E cells. Relative quantitation of duplicate experiments indicated an average of an eightfold increase in transcriptional activity in TGFβ1-treated cells and a 23-fold increase in EGF treated cells after 72 hours. Run on assays were also performed on NRK52E cells after 24 and 48 hours of treating the cells with EGF and TGFβ1. The EGF-treated cells showed a fivefold increase after 24 hours and a 19-fold increase after 48 hours, and the TGFβ1-treated cells showed an eightfold increase in 24 hours and ninefold increase in 48 hours. These data were normalized against the actin gene after subtracting background obtained using pUC7 plasmid as a negative control. These data, as well as the mRNA stability studies,

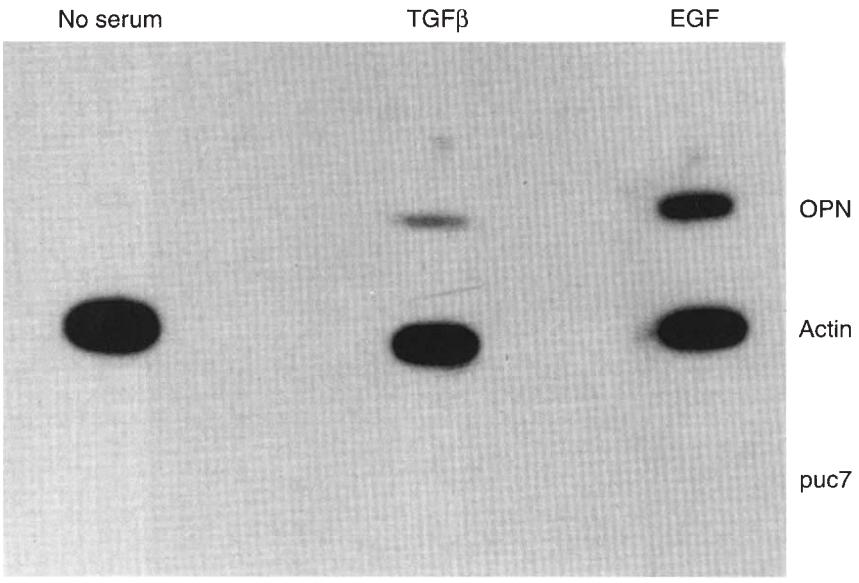

Fig. 6. *Effect of TGFβ1 and EGF on osteopontin gene transcription.* NRK52E cells were treated with the cytokines or control (no serum) for 72 hours and used in nuclear run-on assays as described in the **Methods** section. Radiolabeled nascent transcripts were hybridized to filter-immobilized plasmids containing either osteopontin (opn), actin, or control (pUC7) cDNAs. Results shown are representative of two independent experiments.

suggest that EGF and TGFβ1 act at a transcriptional level to elevate steady-state osteopontin mRNA levels in rat kidney epithelial cells.

Discussion

Osteopontin has been implicated as an important mediator of tubular injury and repair in a number of different acute and chronic renal injury models [11–14, 18, 34]. The mechanism(s) responsible for osteopontin up-regulation during renal disease is unknown, but may involve induction by specific cytokines released by damaged glomeruli, inflammatory cells or injured regions [22]. Our studies suggest that TGFβ1 and EGF may be important regulators of osteopontin synthesis in renal epithelial cells. TGFβ1 and EGF induced both the level of osteopontin mRNA and protein synthesized by a rat renal epithelial cell line, NRK52E cells. These cells, like cortical tubular epithelial cells *in vivo*, normally synthesize very low levels of osteopontin. Using actinomycin D and nuclear run on assays, we found that both TGFβ1 and EGF appear to elevate osteopontin levels secreted by NRK52E cells primarily by enhancing the transcription rate of the osteopontin gene rather than stabilizing the mRNA. Thus, *cis*-acting sequences in the osteopontin gene together with specific *trans*-acting factors most likely regulate osteopontin gene expression in response to TGFβ1 and EGF in rat renal epithelial cells.

In vivo, TGFβ and EGF have both been implicated in renal injury and repair processes. TGFβ has been correlated with glomerular and interstitial fibrosis in experimental models of protein overload proteinuria [13], passive-Heymann nephritis [35], anti-GBM nephritis [36], obstructive uropathy [37], and in human and animal models of diabetic nephropathy [38, 39]. In addition, infusion of anti-TGFβ [40] or expression of decorin [41], a natural inhibitor of TGFβ, suppressed matrix production by glomeruli and buildup of mesangial matrix in rat glomerulonephritis. Most recently, inhibition of TGFβ has been shown to significantly attenuate the characteristic features of diabetic renal involvement, including glomerular hypertrophy and increased matrix expression [42]. Thus, TGFβ appears to be a major regulator of renal glomerular and interstitial fibrosis *in vivo*.

On the other hand, EGF levels decrease in the kidney during mercuric chloride induced acute renal failure [43], and urinary EGF levels decrease in ischemia-induced acute tubular necrosis (ARF) [44] and nephrotoxic ARF [45]. Infusion of EGF enhances recovery from acute renal failure induced by either ischemia [46] or mercuric chloride nephrotoxicity [47], suggesting important roles in renal regeneration mechanisms. Thus, TGFβ and EGF may play distinct, but important roles in regulating the various phases of the renal response to injury. Since osteopontin is a downstream product of all of these cytokines, it is interesting to speculate that it may play an important role in common final pathway(s) required for renal injury and repair processes such as inflammation, fibrosis, and cellular regeneration.

To our knowledge, these are the first studies to demonstrate TGFβ1 and EGF induction of osteopontin mRNA in cultured kidney epithelial cells. TGFβ1 has also been shown to elevate osteopontin mRNA and protein levels in an osteosarcoma cell line, ROS 17/2.8 and normal rat bone cells [48], and rat aortic smooth muscle cells [29]. IGF-I has not been previously reported to regulate osteopontin in any cell system, but has been implicated in renal osteopontin expression *in vivo* [18]. Other cytokines which have been reported to induce osteopontin levels include bFGF in ROS 17/2.8 osteosarcoma cells [49] and rat aortic smooth muscle cells [18], angiotensin II (Ang II) in rat aortic smooth muscle cells [18], PDGF in rat aortic smooth muscle cells [50], LIF in MC3T3-E1 mouse osteoblast cells [51], and IL-1a and TNFα in primary mouse osteoblasts and MC3T3-E1 cells [52].

TGFβ1 and EGF stimulated steady-state levels of osteopontin mRNA, which was reflected in elevated amounts of osteopontin protein secreted by NRK52E cells. Our studies indicate that the mechanisms by which TGFβ1 and EGF up-regulate steady state osteopontin expression in kidney cells occur predominantly at the transcriptional level. These data suggest that *cis*-acting sequences in the osteopontin promoter interacting with specific *trans*-acting factors probably control the elevated steady-state osteopontin mRNA expression observed in response to TGFβ1 and EGF. Transcriptional regulation of the osteopontin gene has also been reported for TGFβ1 [53] in rat osteosarcoma cells. Interestingly,

our data also suggest that other mechanisms besides gene transcription rate and mRNA stabilization may play a role in controlling steady state levels of osteopontin mRNA. We found that TGFβ1 and EGF induced comparable levels of steady state mRNA, despite the finding that the rate of transcription was lower in TGFβ1 treated cells than EGF-treated cells, and TGFβ1 tended to destabilize the osteopontin mRNA compared to EGF treatment. TGFβ1 might enhance splicing of the osteopontin precursor mRNA, stability of the mRNA in the nucleus, or transport to the cytoplasm, thus accounting for the higher steady state levels. The assays perfomed in this study would not permit us to distinguish between these possibilities. TGFβ1 induced destabilization of mRNA is not unique to osteopontin, since TGFβ1 also caused a rapid degradation of c-kit mRNA in FDC-P1 [54]. TGFβ1 also inhibits the expression of iNOs in rat pulmonary aortic smooth muscle cells at both the protein and RNA level [55]. Hence, TGFβ may have effects on several levels of gene expression.

Finally, a number of cytokines failed to induce osteopontin in NRK52E cells. Most surprisingly, Ang II treatment had no effect on osteopontin mRNA or protein levels in these cells, despite the previous finding of Ang II-induced renal osteopontin levels *in vivo* [9]. In that study, infusion of Ang II to rats for two weeks elevated blood pressure and induced severe tubular interstitial disease. The failure of Ang II to induce osteopontin gene expression in cultured renal epithelial cells suggests that the elevated levels following Ang II infusion *in vivo* may have been due to an indirect effect. One possibility is that Ang II-induced renal damage might increase either TGFβ or EGF, thereby leading to increased osteopontin expression. However, we cannot rule out that the NRK 52E cells used in these studies may lack or have few Ang II, IGF, bFGF or PDGF receptors on the cell surface, or are defective in specific cell signalling mechanisms. Consistent with our results, EGF and TGFβ receptors have been previously identified in NRK 52E cells [23, 25, 26]. These possibilities are currently being tested.

Acknowledgments

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Reprint requests to Dr. Cecilia M. Giachelli, Ph.D., Pathology Department, University of Washington, Vascular Biology, Box 357335, Seattle, Washington 98195, USA.
E-mail: ceci@u.washington.edu

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